Protein modification by xanthurenic acid

The purpose of the present invention is the induction of immune response against the pathologies caused by modifications of cellular physiology by the xanthurenic acid. The present invention concerns controlled induction of cellular pathology in the presence of xanthurenic acid. It concerns a formation, *in vitro* or in a cellular system, of the protein covalently modified by xanthurenic acid, but not non-covalent modification described before (Kotake Y. and al., J Biochem. 1975, 77, 685-687; Kobayashi K et al. Chem. Pharm. Bull. 1980,28, 2960-2966). The basis of the invention is an observation of the fact that xanthurenic acid leads to the covalent modification of protein in cells and causes a modification of cellular physiology. Previously, it was described that xanthurenic acid is accumulated in the lens crystalline of the bovine (Malina et al. Graefe's Arch. & Exp. Ophthalmol. 1995, 233, 38-44), and human eye (Malina et al., Graefe's Arch. & Exp. Ophthalmol. 1996, 234, 723-730) with age, and in its presence alpha, beta and gamma -crystallin forms aggregates (ref. as above) which become fluorescent (Malina et al., Eur. J. Ophthalmol. 1996, 6, 250-256). The covalent conjugates are formed by the preparation of the oxidized products of xanthurenic acid, named DOXA, and its reaction with the eye crystallines (Malina et al., Graefe's Arch. & Exp. Ophthalmol. 1996, 234, 723-730). Recently, the experiments showed that xanthurenic acid accumulating in a cell leads to modification of cellular physiology. This modification is due to an accumulation of unfolded proteins. The xanthurenic acid can form covalent bonds with proteins. In the presence of xanthurenic acid which has a yellow color, proteins become yellow. This color persists after electrophoresis of proteins on denaturing gels. These results show that xanthurenic acid is attached to proteins in a covalent way. To change conformation of a protein, it is sufficient to modify an amino acid; many examples are present in the scientific literature. In the presence of xanthurenic acid as indicate it the examples given in this description, several amino acids can be modified. For this reason, the presence of xanthurenic acid in a cell causes an overexpression of the chaperones proteins "named glucose regulated proteins 94 " GRP94. The overexpression of these proteins is known as being caused by the accumulation of unfolded proteins (Kozutsumi, Nature1988, 332, 462-464).

The xanthurenic acid modifies proteins in a random way and this modification concerns also to the proteins chaperones such as for example the GRP 94 and the calreticulin. It was described that these proteins are responsible for the correct conformation of other proteins, and their modification accelerates accumulation of unfolded proteins, among them also unfolded immunoglobulines. These complex modifications of proteins by xanthurenic acid allow the cells to live with a modified physiology. Accumulation of proteins modified by xanthurenic acid in various types of cells (for example cells of the astrocytes, epithelial cells of the lens) causes, for example, an overexpression of the proteases, and a degradation of the calreticulin, a modification of the nuclear factor kappa-B, and an induction of β-amyloid (A4). These results show that xanthurenic acid leads to a cellular pathology by inducing changes of many proteins. The changes observed are proportional to the degree of the protein modification by xanthurenic acid. This new mechanism is caused by the modification of proteins by xanthurenic acid in a cell. In a cell culture of astrocytes an increase in the protein level modified by the xanthurenic acid causes the induction of β-amyloid (A4), which is recognized by the antibody monoclonax from Dako, Denmark, used for the diagnostic of the disease of Alzheimer. The reason of this induction of beta-amyloide (A4) is a modification of the conformation of beta-amyloid precursor protein (APP), due to a modification by xanthurenic acid. The modification gives the signal to an induction of proteases, which degrade the modified APP and induces the formation of beta-amyloid (A4). Xanthurenic acid is an amino acid formed on tryptophan degradation pathway and its accumulation in various types of cells can lead to various pathologies. It can be expected that the animal in which the level of protein modified were increased by xanthurenic acid can be use as model to study effect of drugs. A direct introduction of xanthurenic acid per os way or other ways, can be used as a model of development of the disease of Alzheimer, prion diseases, senile cataract, atherosclerosis, rheumatisms, or degeneration of the retina with age.

The fact that xanthurenic acid causes a deregulation of cellular physiology allows a controlled induction of cellular pathology. Proteins modified by xanthurenic acid and injected into an animal will induce an immune response against unfolded proteins. The pathological cells are not eliminated because of the modification of the immune system by xanthurenic acid followed by degradation of the proteins chaperones like the GRP94. The induction of the immune response against unfolded proteins can prevent the pathological effect which takes place with the formation of these proteins during aging. The vaccines based on proteins modified by xanthurenic acid will have a preventive role against the diseases induced by such a modified proteins. The proteins modified by the xanthurenic acid can be introduced to mammals by using all non-toxic solvents in which they are soluble. Degrees of the protein modifications by the xanthurenic acid, and quantity of protein to be introduced will depend on protein expected to be modified and the aim to achieve by vaccination. Fragments of proteins, peptides or the synthetic sequences can be used to form products combined with xanthurenic acid. These compounds are introduced into a mammal to induce an immune response.

Example 1. Formation of the protein modified by xanthurenic acid in the culture of epithelial cells. The primary culture of the epithelial cells of bovines in minimal essential medium (MEM) was treated by xanthurenic acid. Xanthurenic acid was added to this medium at concentration 0, 1, 2, 4 mM. After 24 hours of culture the cells were washed by using a buffer PBS (5 mM sodium phosphates,150 mM NaCl, pH 7.1) and lysed in a buffer containing 50 mM Tris-HCl (pH8), 150 mM NaCl, 100mg/ml PMSF, 1% Triton X-100. The extracts were applied to a column of Sephadex G-50 and were eluted by using 0,005 M $NaHCO_3$. Xanthurenic acid was quantified in the protein extracts by UV spectrometry. The concentration of proteins was calculated using a standard curve of the absorption of the known quantities of bovine albumin having the molecular weight of 67,5 kD after an incubation with xanthurenic acid λ=342 nm ($E_{\lambda max}$ 6 500 according to Merck Index, Merck and Co. edition White House Station, New York, 1996). The concentration of xanthurenic acid

corresponded respectively, to 0, 1, 3, 9 moles per mole of proteins. The analysis of proteins after a transfer from SDS-PAGE gel onto a nylon membrane (Western blot) in the presence of the various antibodies showed that in the presence of proteins modified by xanthurenic acid the level of nuclear factor -kappaB, beta-amyloid (A4), and calpain Lp82 was changed.

Example 2. Formation of the proteins modified by xanthurenic acid in a cellular culture of astrocytes. The culture of astrocytes of rat in medium MEM was treated with xanthurenic acid at concentration 0, 2, 4, 8 mM. The concentration of xanthurenic acid (XA) in extracts was calculated as in example 1, and corresponded respectively to the 0; 1 mole XA per 8 moles of proteins; 3 moles of XA per 2 moles of proteins; 1 mole XA per 5 moles of proteins. In the presence of proteins modified by xanthurenic acid, the nuclear factor-kappaB had molecular weights of 50 kD, 52kD, and 55 kD instead of the normal size 50 kD. The beta-amyloid formation (A4), which was not detectable without the presence of xanthurenic acid, was strongly induced. These results showed that the increase of xanthurenic acid in the cell will cause a deregulation of cellular physiology. These results show that it is possible to induce a cellular pathology artificially by increasing in a cell the level of proteins modified by xanthurenic acid. This new mechanism described is caused by the covalent modification of proteins by xanthurenic acid.

Example 3. Formation of the proteins modified by the xanthurenic acid in a cellular extract of the retina. Xanthurenic acid with 0, 2, 4, 8 mM was incubated with protein extracts of the retina during one week and the extracts were treated as described in example 1, and the concentrations corresponded, respectively, to the 0; 2 mole XA per 1 mole of proteins; 3 moles of XA per 1 mole of proteins; 5 moles XA per moles of proteins.

Example 4. Formation of the proteins modified by xanthurenic acid in the tissues culture. The crystalline of pig lenses was incubated in 0 and 2 mM solutions of xanthurenic acid for one week. Xanthurenic acid diffused to the lens crystalline. The cortex of the lens crystalline was homogenized in a buffer phosphates (PBS) of 7.4. The non-soluble part of proteins was separated by centrifugation with 10 000g. The concentration of proteins was measured at 280 nm, the insoluble parts of proteins were dissolved in 4 mM urea or 8 mM. Xanthurenic acid was present in all of the

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Example 5. Preparation of xanthurenic acid conjugates with proteins of bacteria. The mycelium of Streptomyces incarnatus, a Gram-positive, mycelial bacteria, was cultivated in the absence or in the presence of 2 mM of xanthurenic acid. 100 ml of each culture were suspended in the phosphate buffer at concentration 0.05 M, pH 7, contained 0.1% of beta -mercaptoethanol. The suspension was frozen by incubation in a bath with dry ice-methanol. The frozen cells were broken in Hinton-press with a pressure of 360 atmospheres. The proteins of cytosol were separated from the membranes fraction by centrifugation at 100 000g for one hour. The solution was treated by the addition of 2,5 % of streptomycine to precipitate the nucleic acids, which were eliminated by centrifugation at 5000g for 10 min. The concentrations of xanthurenic acid in the proteins were measured as described in Exemple 1. The concentrations of xanthurenic acid in proteins corresponded to 0 and 0.5 mole of xanthurenic acid per a mole of proteins.

Example 6. Induction of an immune response against protein modified by xanthurenic acid. The calreticulin is modified by xanthurenic acid in a cell and partially degraded. 3 mg of calreticulin in the sterile phosphate buffer of pH 7,4 were incubated with 4 mM of xanthurenic acid for 72 hours, at room temperature. The modified calreticulin was injected into mice. Six mice (weighting about 100 g) were immunized by subcutaneous injection of the same quantities of 500 micrograms of

calreticulin. Another group of mouse was not immunize. The immunization was repeated three times in the two weeks interval. The calreticulin was analyzed in the plasma of the animals after three months. Proteins of plasma of mouse were analyzed by electrophorese on a denaturing gel (Laemmli, Nature 1970, 227, 680- 685).

The proteins were transferred on a Nylon membrane. The detection of the calreticulin was carried out by using an antibody against the calreticulin. In the plasma of non-immunized mouse, the degraded calreticulin presented a molecular weight of 55 kD instead of 63kD. The immunized mice plasma contained 60 degraded percent less of degraded calreticulin. This way can be used to delay the pathological aging of the cells due to a modification of the conformation of proteins, among them of the proteins chaperones. The injections of proteins modified by xanthurenic acid can have a preventive effect against pathologies associated with aging. A immunotherapie using the monoclonal antibodies would be possible to delay the effect of unfolded proteins. For example an antibody against the amyloid precursor protein modified by xanthurenic acid is supposed to delay the development of the Alzheimer's disease.